

JCO3 Rec'd PCT/PTO 22 JAN 2002

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER 111737
		U.S. APPLICATION NO. (if known, sec 37 C.F.R.1 5) 10/031439
INTERNATIONAL APPLICATION NO. PCT/FR00/02202	INTERNATIONAL FILING DATE July 31, 2000	PRIORITY DATE CLAIMED July 30, 1999
TITLE OF INVENTION METHOD FOR IN VITRO CULTURE OF VIRUSES OF THE TOGAVIRIDAE AND FLAVIVIRIDAE FAMILIES AND USES		
APPLICANTS FOR DO/EO/US Patrice ANDRE; Vincent LOTTEAU; Glaucia PARANHOS-BACCALA; Florence KOMURIAN-PRADEL		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<p>1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).</p> <p>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p> <p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</p> <p>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p> <p>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input type="checkbox"/> have been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).</p> <p>Items 11. to 16. below concern other document(s) or information included:</p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p><input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>14. <input type="checkbox"/> A substitute specification.</p> <p>15. <input type="checkbox"/> Entitlement to small entity status is hereby asserted.</p> <p>16. <input type="checkbox"/> Other items or information:</p>		

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) 10/031439		INTERNATIONAL APPLICATION NO. CT/FR00/02202		ATTORNEY'S DOCKET NUMBER 111737	
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17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National fee (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO\$890.00 International preliminary examination fee paid to USPTO (37 CFR1.482)\$710.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))\$740.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO\$1,040.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)\$ 100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =	CALCULATIONS	PTO USE ONLY

Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$	
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Claims	Number Filed	Number Extra	Rate		
Total Claims	23 - 20 =	3	X \$ 18.00	\$54.00	
Independent Claims	3 - 3 =	0	X \$ 84.00	\$	
Multiple dependent claim(s)(if applicable)			+ \$280.00	\$	
TOTAL OF ABOVE CALCULATIONS =				\$944.00	
Reduction by 1/2 for filing by small entity, if applicable.				-	\$
SUBTOTAL =				\$	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 month from the earliest claimed priority date (37 CFR 1.492(f)).				+	\$
TOTAL NATIONAL FEE =				\$944 00	
				Amount to be refunded	\$
				Charged	\$

a. ☒ Check No. 127072 in the amount of \$944.00 to cover the above fees is enclosed.

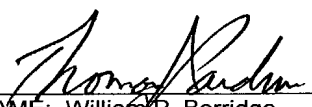
b. ☐ Please charge my Deposit Account No. _____ in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Director is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 15-0461. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Date: January 22, 2002


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 REGISTRATION NUMBER: 30,411

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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of

Patrice ANDRE; Vincent LOTTEAU; Glaucia
PARANHOS-BACCALA; Florence KOMURIAN-
PRADEL

Application No.: U.S. National Stage of
PCT/FR00/02202

Filed: January 22, 2002

Docket No.: 111737

For: METHOD FOR IN VITRO CULTURE OF VIRUSES OF THE TOGAVIRIDAE AND
FLAVIVIRIDAE FAMILIES AND USES

PRELIMINARY AMENDMENT

Director of the U.S. Patent and Trademark Office
Washington, D. C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE ABSTRACT:

Please add the Abstract as attached hereto.

IN THE CLAIMS:

Please replace claims 1-4, 6, 8, 10, 13, 14, 17, 19, 20 and 24 as follows:

1. (Amended) A method for culturing, propagating and replicating, *in vitro*, viruses belonging to the *Togaviridae* or *Flaviviridae* families, according to which there is at least one LVP fraction obtained from serum or from plasma of a patient infected with at least one virus belonging to the *Togaviridae* or *Flaviviridae* families, and said fraction is brought into contact with permissible cells for a predetermined period of time in a suitable culture medium containing an activating agent chosen from unsaturated fatty acid, or a derivative of an

unsaturated fatty acid, said fatty acid comprising from 16 to 20 carbon atoms or a mixture thereof.

2. (Amended) A method for culturing, propagating and replicating, *in vitro*, viruses belonging to the *Togaviridae* or *Flaviviridae* families, according to which there is at least one LVP fraction, associative with human immunoglobulins, obtained from serum or from plasma of a patient infected with a least one virus belonging to the *Togaviridae* or *Flaviviridae* families, and said fraction is brought into contact with permissible cells for a predetermined period of time in a suitable culture medium containing an activating agent chosen from an unsaturated fatty acid, or a derivative of an unsaturated fatty acid, said fatty acid comprising from 16 to 20 carbon atoms or a mixture thereof.

3. (Amended) The method as claimed in claim 1, in which the receptor for lipoproteins is the LSR and /or the surface receptor from LDLs.

4. (Amended) The method as claimed in claim 1, in which the unsaturated fatty acid is chosen from oleic acid, palmitoleic acid, linoleic acid, linolenic acid, arachidonic acid, transhexadecenoic acid and elaidic acid, or derivatives thereof.

6. (Amended) The method as claimed in claim 1, in which the permissive cells are primary human or animal hepatocyte cells, cells chosen from the human or animal hepatocarcinoma cell line group, dendritic cells, macrophage cells, Kupffer cells and combinations thereof which may or may not be associated with lymphocytes.

8. (Amended) The method as claimed in claim 1, in which the culture medium comprises, besides the ingredients required for culturing and the fatty acid or the derivative of fatty acid, an apoptosis-modulating agent.

10. (Amended) The method as claimed in claim 1, in which the medium is DMEM medium, or a medium derived from DMEM medium, RPMI medium or a derivative of RPMI medium.

13. (Amended) The method as claimed in claim 1, in which, after bringing the permissive cells and said LVP fraction into contact, said permissive cells thus infected under conditions as defined according to claim 1 are subcultured several times and the presence of said virus is demonstrated in the said permissive cells by RT-PCR and/or by an immunological technique, such as by indirect immunofluorescence in particular using an antibody specific for said virus and/or by flow cytometry.

14. (Amended) The method as claimed in claim 1, in which the virus belongs to the Flaviviridae family and to the Hepacivirus genus.

17. (Amended) A method for preparing a composition for detecting, in a sample, antibodies directed against at least one virus belonging to the *Togaviridae* or *Flaviviridae* families, which comprises at least one partial or total purification of the viral particles of said virus or of the polypeptides obtained in using a culturing method as claimed in claim 1.

19. (Amended) A method for obtaining antibodies or antibody fragments directed against at least one virus belonging to the *Togaviridae* or *Flaviviridae* families, according to which an animal is immunized with viral particles or polypeptides obtained using a culturing method as claimed in claim 1.

20. (Amended) A diagnostic composition comprising at least the viral particles according to the method defined in claim 17.

24. (Amended) A method for screening and/or selecting at least one antiviral molecule, according to which infected permissive cells are obtained in accordance with claim 1 and said antiviral molecule is brought into contact with said infected permissive cells.

Please cancel claim 16 without prejudice to or disclaimer of the subject matter contained therein.

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REMARKS

Claims 1-15 and 17-24 are pending. By this Preliminary Amendment, claim 16 is cancelled without prejudice to or disclaimer of the subject matter contained therein. Claims 1-4, 6, 8, 10, 13, 14, 17, 19, 20 and 24 are amended. Prompt and favorable examination on the merits is respectfully requested.

The attached Appendix includes marked-up copies of each rewritten claim (37 C.F.R. 1.121(c)(1)(ii)).

Respectfully submitted,

Thomas Hardin

William P. Berridge
Registration No. 30,411

Thomas J. Pardini
Registration No. 30,411

WPB:TJP/kaf

Attachments:
Appendix
Abstract

Date: January 22, 2002

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DEPOSIT ACCOUNT USE
AUTHORIZATION
Please grant any extension
necessary for entry;
Charge any fee due to our
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APPENDIX

Changes to Claims:

1. (Amended) A method for culturing, propagating and replicating, *in vitro*, viruses belonging to the *Togaviridae* or *Flaviviridae* families, according to which there is at least one LVP fraction obtained from serum or from plasma of a patient infected with at least one virus belonging to the *Togaviridae* or *Flaviviridae* families, and said fraction is brought into contact, ~~for a predetermined period of time in a suitable culture medium,~~ with permissible cells for a predetermined period of time in a suitable culture medium containing which have ~~an endocytosis pathway relayed by at least one receptor for lipoproteins and modulated by an~~ activating agent chosen from unsaturated fatty acid, or a derivative of an unsaturated fatty acid, said fatty acid comprising from 16 to 20 carbon atoms or a mixture thereof.
2. (Amended) A method for culturing, propagating and replicating, *in vitro*, viruses belonging to the *Togaviridae* or *Flaviviridae* families, according to which there is at least one LVP fraction, associative with human immunoglobulins, obtained from serum or from plasma of a patient infected with a least one virus belonging to the *Togaviridae* or *Flaviviridae* families, and said fraction is brought into contact, ~~for a predetermined period of time in a suitable culture medium,~~ with permissible cells for a predetermined period of time in a suitable culture medium containing which have an endocytosis pathway relayed by at least one receptor for lipoproteins and modulated by ~~an activating agent chosen from an~~ unsaturated fatty acid, or a derivative of an unsaturated fatty acid, said fatty acid comprising from 16 to 20 carbon atoms or a mixture thereof.
3. (Amended) The method as claimed in claim 1 ~~or 2~~, in which the receptor for lipoproteins is the LSR and /or the surface receptor from LDLs.
4. (Amended) The method as claimed in ~~any of the preceding claims~~ claim 1, in which the unsaturated fatty acid is chosen from oleic acid, palmitoleic acid, linoleic acid,

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linolenic acid, arachidonic acid, transhexadecenoic acid and elaidic acid, or derivatives thereof.

6. (Amended) The method as claimed in ~~any one of the preceding claims~~ claim 1, in which the permissive cells are primary human or animal hepatocyte cells, cells chosen from the human or animal hepatocarcinoma cell line group, dendritic cells, macrophage cells, Kupffer cells and combinations thereof which may or may not be associated with lymphocytes.

8. (Amended) The method as claimed in ~~any one of the preceding claims~~ claim 1, in which the culture medium comprises, besides the ingredients required for culturing and the fatty acid or the derivative of fatty acid, an apoptosis-modulating agent.

10. (Amended) The method as claimed in ~~any one of the preceding claims~~ claim 1, in which the medium is DMEM medium, or a medium derived from DMEM medium, RPMI medium or a derivative of RPMI medium.

13. (Amended) The method as claimed in ~~any one of preceding claims~~ claim 1, in which, after bringing the permissive cells and said LVP fraction into contact, said permissive cells thus infected under conditions as defined according to ~~any one of the preceding claims~~ claim 1 are subcultured several times and the presence of said virus is demonstrated in the said permissive cells by RT-PCR and/or by an immunological technique, such as by indirect immunofluorescence in particular using an antibody specific for said virus and/or by flow cytometry.

14. (Amended) The method as claimed in ~~any one of the preceding claims~~ claim 1, in which the virus belongs to the Flaviviridae family and to the Hepacivirus genus.

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17. (Amended) A method for preparing a composition for detecting, in a sample, antibodies directed against at least one virus belonging to the *Togaviridae* or *Flaviviridae* families, which comprises at least one partial or total purification of the viral particles of said virus or of the polypeptides obtained in using a culturing method as claimed in claim 1 ~~or 2~~.

19. (Amended) A method for obtaining antibodies or antibody fragments directed against at least one virus belonging to the *Togaviridae* or *Flaviviridae* families, according to which an animal is immunized with viral particles or polypeptides obtained using a culturing method as claimed in claim 1 ~~or 2~~.

20. (Amended) A diagnostic composition comprising at least the viral particles ~~or the polypeptides obtained~~ according to the method defined in ~~either of claims 17 and 18 or the antibodies obtained according to the method defined in claim 19~~ claim 17.

24. (Amended) A method for screening and/or selecting at least one antiviral molecule, according to which infected permissive cells are obtained in accordance with claim 1 and said antiviral molecule is brought into contact with ~~an infected cell line~~ said infected permissive cells.

Claim 16 is canceled.

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Method for in vitro culture of viruses of the
Togaviridae and Flaviviridae families and uses

5 The present invention in particular relates to a method
for culturing and propagating *in vitro*, an RNA virus
involved in the development of human viral pathologies.

10 The culturing and propagating method of the invention
applies mainly to viruses belonging to the *Togaviridae*
and *Flaviviridae* families. These viruses have the
common characteristics of being enveloped, non-
segmented, positive-sense, single-stranded RNA viruses.

15 The *Togaviridae* family comprises the viruses of the
Alphavirus and *Rubivirus* genera. The virions are 70 nm
in diameter, are spherical and comprise an envelope and
peplomers composed of a heterodimer of two
glycoproteins. The genome consists of a single molecule
of single-stranded RNA of 9.7 to 11.8 kb. The
20 structural proteins include a protein of the capsid and
two envelope glycoproteins. The virions contain lipids
derived from the hosts' cell membrane. Replication
involves the synthesis of a complementary RNA strand
which is used as a matrix for the synthesis of the
25 genomic RNA. The genomic RNA is used as a matrix for an
intermediate replication RNA, which, in turn, is used
as a matrix for the synthesis of mRNA and the
production of a polyprotein precursor which is cleaved
in order to obtain structural and nonstructural
30 proteins. Replication takes place in the cytoplasm and
assembly involves budding through the membrane.

35 The Sindbis virus, the equine encephalitis viruses
(western, eastern, Venezuelan), the chikungunya virus,
the o'nyong-nyong virus, the Igbo Ora virus, the Ross
River virus, the Mayaro virus and the Barmah Forest
virus belong to the *Alphavirus* genus and are agents
which are pathogenic to humans. The hepatitis E virus

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GBV-C. It has a genomic structure close to that of the
flaviviruses. Its 2900 amino acid polyprotein is
encoded by a 9,400 nucleotide RNA, the 5' end of which
encodes structural proteins (truncated nucleocapsid and
5 envelope) and the 3' end of which encodes proteins
which have a role in replication. It is transmitted by
blood transfusion and can be detected in patients who
have acute, chronic and violent forms of hepatitis. Its
contribution in acute and chronic liver diseases is
10 still poorly understood. GBV-A and GBV-B have also been
described and are very close to HGV.

Particles containing viral RNA, very heterogeneous in
density, are found in the plasma of patients infected
15 with HCV. This heterogeneity in density of the
particles containing viral RNA is attributed to their
association in variable proportion with lipoproteins
(Thomsen et al., 1993, Med. Microbiol. Immunol. 182:
639). In the description of the present patent
20 application, the inventors have named these hybrid
particles LVPs (lipo-viro-particles). The distribution
of each of these forms along a density gradient varies
from one patient to the other. Existing analyses of low
density particles show densities covering those of the
25 LDLs (low density lipoproteins) and of the VLDLs (very
low density lipoproteins). The size described (50 nm)
makes them close to the VLDLs. Moreover, the particles
may be precipitated (sometimes even in their entirety,
by anti- β -lipoprotein sera (Thomsen et al., 1992, Med.
30 Microbiol. Immunol. 181: 293; Prince et al., 1996, J.
Viral. Hepat. 3: 11).

It has also been observed that the hepatitis G virus
may be precipitated, virtually in its entirety, by
35 anti- β -lipoprotein sera obtained from patients
chronically infected with HGV (Sato et al. Biochem.
Biophys. Res. Commun. 229: 719).

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It has, moreover, been observed that infection of human hepatocytes, with HCV, is accompanied by intracytoplasmic accumulation of lipid vesicles. Transfection of the HCV gene encoding the capsid into a continuous line of human hepatocytes (Hep/G2) induces the same phenomenon (Barba et al., 1997, PNAS 94:1200). These vesicles are rich in triglycerides. The HCV capsid protein is arranged side by side at the surface of the vesicles, as is the apolipoprotein apo A-II.

The apolipoproteins allow lipids to be transported by introducing structural stability and solubility to the lipoprotein particles with which they are associated. They also determine the outcome for these particles within the metabolism, in particular by targeting them onto receptors.

Apo B 100 is the main apolipoprotein of VLDLs, LDLs and IDLs (intermediate density lipoproteins). It is synthesized in the liver and is essential for the assembly and secretion of VLDLs from the liver. It also constitutes a ligand for binding LDLs to their receptors. One of the receptors for LDLs, the LDL receptor, is a cell surface protein which binds and internalizes the lipoproteins rich in cholesterol which contain apo B 100 and apo E. Apo E is mainly synthesized in hepatocytes. It constitutes a ligand for binding various lipoproteins to the receptor for LDLs and to the LRP (LDL-receptor-related protein).

Apo B 48 is essential for the assembly and secretion of chylomicrons. It is encoded by the same gene and the same mRNA as apo B 100, but in the intestine, a mutation introduces a stop codon such that apo B 48 contains only 40% of the total length of apo B 100. Its role in the metabolism of chylomicrons in the plasma is poorly understood, but the individuals who exhibit mutations which interfere with its normal synthesis do

not have any, or have very low levels of, chylomicrons, VLDLs, IDLs and LDLs.

The nature of the abovementioned LVPs containing viral
 5 RNA is not, to date, known, but the present inventors
 have put forward the hypothesis and verified that at
 least one of the cell surface receptors for
 lipoproteins contributes to the infection and
 propagation of viruses belonging to the *Togaviridae* and
 10 *Flaviviridae* families, in a cell culture. They have, in
 fact, shown that the LVPs are ligands for a receptor-
 associated endocytosis pathway, preferentially for the
 LSR (lipolysis-stimulated receptor), (Frances T. Yen et
 al., *Biochemistry*, 1994, 33, 1172-1180 and Bernard E.
 15 Bihain and Frances T. Yen, *Biochemistry*, 1992, 31,
 4628-4636). They have, moreover, shown that the LVPs
 may be associated with human immunoglobulins.

On this basis, they have developed novel methods which
 20 allow the culturing, propagating and replicating, *in*
vitro, of viruses belonging to the *Togaviridae* and
Flaviviridae families. These methods, which make it
 possible to obtain propagation of these viruses, are
 useful in particular for studying their mechanisms of
 25 replication, for testing neutralising and antiviral
 antibodies, and for developing biological materials for
 diagnosis and therapy. Moreover, the methods of the
 invention make it possible to obtain an infected cell
 line which is useful for screening and/or selecting at
 30 least one antiviral molecule, by bringing the infected
 cell line and the antiviral molecule into contact.

According to this method, there is at least one LVP
 fraction or at least one LVP fraction associated with
 35 the human immunoglobulins, obtained from serum or from
 plasma of the patient infected with a virus belonging
 to the *Togaviridae* or *Flavivirida* family, and said LVP
 fraction is brought into contact, for a predetermined
 period of time in a suitable culture medium, with

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permissible cells which have an endocytosis pathway relayed by at least one receptor for lipoproteins and modulated by an activating agent chosen from unsaturated fatty acids, derivatives of unsaturated
5 fatty acids comprising from 16 to 20 carbon atoms and mixtures thereof.

The receptor for lipoproteins is the LSR and/or the surface receptor for LDLs, and advantageously said
10 permissive cells have both receptors.

The unsaturated fatty acid is chosen from oleic acid, palmitoleic acid, linoleic acid, linolenic acid, arachidonic acid, transhexadecenoic acid and elaidic
15 acid, or derivatives thereof. In a preferred embodiment of the invention, the fatty acid selected is oleic acid, which is added to the culture medium at a concentration of between 0.1 and 1 mM, preferably 0.5 mM.

20 The cells are preferably cells chosen from human or animal hepatocyte cells, the human or animal hepatocarcinoma cell line group, dendritic cells, macrophage cells, Kupffer cells and combinations
25 thereof which may or may not be associated with lymphocytes. Advantageously, these cells are human hepatocarcinoma cells of the PLC/PRF/5 cell line.

Preferentially, the culture medium comprises, besides
30 the ingredients required for culturing and the activating agent, at least one apoptosis-modulating agent. This apoptosis-modulating agent is chosen from interferons, anti-interferons, in particular anti-alpha and beta interferons; anti-caspases 3, in particular
35 peptide analogs, such as z-VADfmk, i.e. N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, and antibodies directed against anti-caspases 3.

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In fact, the present inventors have observed that, in patients infected both with HIV and with *Hepacivirus* HCV, taking certain HIV protease inhibitors, such as Ritonavir (commercial name), causes an increase in the
 5 HCV virema associated with hepatic cytolysis. The observation of hepatic cytolysis immediately after taking a medicinal product which, in addition to its antiprotease activity, also modifies the regulation of apoptosis, is suggestive of induction of the signal for
 10 apoptosis. For this reason, it is advantageous to add to the method of the invention at least one apoptosis-modulating agent.

The suitable medium selected is in particular chosen
 15 from DMEM medium, or a medium derived from DMEM medium, and RPMI medium, or a medium derived from RPMI medium. Preferably, it is a medium derived from DMEM medium which comprises the complete DMEM medium marketed by the company Gibco BRL, supplemented with from 0 to 10
 20 mM of sodium pyruvate, 0 to 10% of nonessential amino acids, 1 to 10 mM of glutamine, 100 to 200 U/ml of penicillin, 100 to 200 mg/ml of streptomycin and 1 to 20% of calf serum.

25 This medium advantageously also comprises 0.1 to 0.5% of BSA (bovine serum albumin) or of HSA (human serum albumin) coupled to a fatty acid, according to the method of Dixon et al., 1991, Journal of Biological Chemistry, vol. 226, p. 5080.

30 In a preferred embodiment of the method of the invention, prior to bringing the permissive cells into contact with the LVP fraction, they are washed in a PBS buffer preheated to a temperature of approximately
 35 37°C.

The permissible cells thus infected under the conditions described above are subcultured several times and the presence of said virus is demonstrated in

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the infected permissible cells and in the culture supernatant by RT-PCR and/or by an immunological technique, such as indirect immunofluorescence using an antibody specific to said virus, and/or by flow
5 cytometry.

The method for culturing, propagating and replicating described above is particularly suitable for culturing, propagating and replicating the viruses belonging to
10 the *Flaviviridae* family and to the *Hepacivirus* genus, in particular the hepatitis C and hepatitis G viruses, including the GBV-A, GBV-B and GBV-C viruses, for which, to date, no effective culturing method existed.

15 The subject of the invention is also a culture medium for culturing viruses belonging to the *Togaviridae* and *Flaviviridae* families, which medium comprises the complete DMEM medium marketed by the company Gibco BRL, supplemented with 0 to 10 mM of sodium pyruvate, 0 to
20 10% of nonessential amino acids, 1 to 10 mM of glutamine, 100 to 200 U/ml of penicillin, 100 to 200 mg/ml of streptomycin and 1 to 20% of calf serum. This medium advantageously also comprises 0.1 to 0.5% of BSA (bovine serum albumin) or of HSA (human serum albumin)
25 coupled to a fatty acid, according to the method given by way of reference above.

A subject of the invention is also a cell line infected with at least one virus belonging to the *Togaviridae*
30 and *Flaviviridae* families, in which the cells in the cell line are permissible cells which have an endocytosis pathway relayed by at least one receptor for lipoproteins, which can be modulated, inter alia, by an activating agent, said cells being capable of
35 propagating and replicating the virus. Preferentially, the receptor is the LSR and/or the receptor for LDLs, and advantageously the permissive cells have both receptors.

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In particular, the cell line is a line in which the cells are derived by infecting a cell chosen from primary human or animal hepatocyte cells, cells of the human and animal hepatocarcinoma cell line group, in particular a cell originating from the human hepatocarcinoma cell line PLC/PRF/5 which has a surface receptor for lipoproteins which can be activated by fatty acids, dendritic cells, macrophage cells and Kupffer cells. However, the invention is not limited to a cell of a cell line which has at least the LSR surface receptor for lipoproteins, and encompasses the transfected or transformed cells which are capable of expressing, at their surface, the LSR receptor and/or the receptor for LDLs. The infected cell line obtained according to the method of the invention can be used for screening and/or selecting at least one antiviral molecule, according to which the antiviral molecule and the infected cell line are brought into contact.

The invention also relates to a method for preparing a composition for detecting, in a sample, antibodies directed against at least one virus belonging to the *Togaviridae* and *Flaviviridae* families, which comprises at least one partial or total purification of the viral particles of said virus or of the polypeptides obtained using one of the methods as defined above. In particular, said viral particles or said polypeptides are attached to a solid support.

Moreover, the invention relates to a method for obtaining antibodies or antibody fragments directed against at least one virus belonging to the *Togaviridae* and *Flaviviridae* families, according to which an animal is immunized with viral particles or polypeptides obtained using one of the methods as defined above. The production of polyclonal and monoclonal antibodies or of antibody fragments is part of the general knowledge of those skilled in the art. By way of example, mention may be made of Köhler G. and Milstein C. (1975),

al., 1997, Journal of Immunology 159: 5821-5833 and
Bird et al., 1988, Science 242: 423-426) of a native
antibody, and the term "derivative" is intended to
mean, inter alia, a chimeric derivative of a native
5 antibody (see, for example Arakawa et al., 1996, J.
Biochem 120: 657-662 and Chaudray et al., 1989, Nature
339: 394-397).

The invention also relates to a diagnostic composition
10 comprising at least the viral particles of the
polypeptides obtained according to the method of the
invention, or the antibodies obtained according to the
method defined above, and a diagnostic kit comprising,
inter alia, said composition, and also an immunization
15 composition comprising at least the viral particles or
the polypeptides optionally associated with a
pharmaceutically acceptable vehicle and/or excipient
and/or adjuvant. However, the invention also opens up
other therapeutic perspectives in that it makes
20 possible to develop a therapeutic composition capable
of qualitatively and/or quantitatively influencing the
propagation and replication of the abovementioned
viruses *in vivo*, since it comprises, inter alia, a
ligand capable of modulating, of repressing or of
25 inhibiting the endocytosis pathway relayed by at least
one of the receptors for lipoproteins, the ligand being
chosen from an antagonistic antibody directed against
said receptor, the production of which is within the
scope of those skilled in the art, as described above,
30 and an unnatural protein, i.e. a soluble protein
obtained by genetic recombination, or a soluble
synthetic polypeptide, which binds to said receptor, or
in that it comprises, inter alia, at least one molecule
which modulates, represses or inhibits the expression
35 of the gene encoding said receptor or the activity of
the promoter which encodes said receptor.

Finally, the subject of the invention is a method for
screening and/or selecting at least one antiviral

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molecule, according to which said antiviral molecule and an infected cell line obtained according to one of the methods of the invention are brought into contact.

5 The term "virus belonging to the *Togaviridae* and *Flaviviridae* families", as used in the invention, refers to any viral species, among which are strains pathogenic for humans, variant strains, attenuated strains and defective strains derived from said
10 strains. In fact, RNA viruses are known to have a high spontaneous mutation rate. Multiple strains may, therefore, exist which may be more or less virulent. It is within the scope of those skilled in the art to identify such strains, for example by homology of
15 nucleic acid and/or peptide sequences compared to a reference strain, and/or by identifying a strain or an isolate with respect to morphological and/or immunological criteria.

20 The term "cell line" refers to a culture of permissive cells in which the virus propagates after a first culturing, and therefore comprises, but is not limited to, the individual cells, the harvested cells and the cultures containing the cells in so far as they are
25 derived from cells of the reference cell line. It is known that spontaneous or induced changes may occur in the caryotype during storage or transfer. Therefore, the cells derived from the reference cell line may not be strictly identical to the cells or cultures of
30 origin, and the "cell line" also refers to the variants. The term "cell line" also includes immortalized cells.

The term "permissive cells" refers to all cells which
35 are capable of propagating the virus, i.e. cells which have an endocytosis pathway relayed by at least one receptor for lipoproteins.

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An immunization composition is a composition which comprises at least the viral particles or the polypeptides obtained according to the method of the invention, but is not limited to them, and also covers
5 the recombinant proteins and fragments thereof which may be obtained using techniques of genetic recombination in a suitable host cell. Such an immunization composition may comprise, if necessary, a vehicle and/or an excipient and/or an adjuvant, on the
10 condition that they are pharmaceutically acceptable.

The term "pharmaceutically acceptable vehicle" is intended to mean the supports and vehicles which can be administered to humans or to animals, as described for
15 example in Remington's Pharmaceutical Sciences 16th Ed., Mack Publishing Co. The pharmaceutically acceptable vehicle is preferably isotonic or hypotonic or exhibits weak hypertonicity and a relatively low ionic strength. The definitions of the pharmaceutically
20 acceptable excipients and adjuvants are also given in Remington's Pharmaceutical Sciences, mentioned above.

Example 1: Preparation of the biological material

25 The lipoproteins are separated from the plasma or from the serum of a patient who has not eaten for 12 hours and who has been detected as positive for the hepatitis C virus.

30 1% of an EDTA solution (0.15 M NaCl - 0.1 M EDTA) is added to the blood taken from the patient. The mixture is centrifuged for 10 minutes at 3500 rpm, at the temperature of 4°C. The plasma is then harvested and stored at 4°C until used.

35 The patient's blood is harvested onto a dry tube and, after clotting, centrifuged for 10 minutes at 3000 rpm, at a temperature of 4°C. The serum is removed and stored at 4°C until used.

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(i) Production of a fraction comprising LVPs with a density of less than 1.0063 g/ml, of a fraction comprising LVPs with a density of between 1.0063 g/ml and 1.063 g/ml, and of a fraction comprising LVPs of greater than 1.063 g/ml.

The plasma and the serum are, respectively, ultracentrifuged for 4 hours at 100,000 rpm, at 4°C in a TL100 machine marketed by the company Beckman and comprising a TL100.4 rotor. The upper fraction which contains the LVPs with a density of less than 1.0063 g/ml is recovered and stored at 4°C. The lower fraction is adjusted to a density of 1.063 g/ml by adding 7.21 g of NaBr per 100 ml of the fraction. The lower fraction is then ultracentrifuged for 4 hours at 100,000 rpm, at 4°C, in a TL100 machine comprising a TL100.4 rotor. The resulting upper fraction containing the fraction with a density of between 1.0063 g/ml and 1.063 g/ml is recovered and stored at 4°C.

(ii) Reduction of a fraction comprising LVPs with a density of less than 1.025 g/ml, of a fraction comprising LVPs with a density of between 1.025 g/ml and 1.063 g/ml, and of a fraction comprising LVPs with a density of greater than 1.063 g/ml.

The plasma and the serum are, respectively, adjusted to a final density of 1.025 g/ml by adding 2.518 g of NaBr per 100 ml. Centrifugation is carried out for 4 hours at 100,000 rpm, at 4°C, on the TL100 machine comprising a TL100.4 rotor.

The upper fraction which contains the fraction with a density of less than 1.025 g/ml is recovered and stored at 4°C. The lower fraction is just adjusted to a density of 1.063 g/ml by adding 4.84 g of NaBr per 100 ml. The lower fraction is then ultracentrifuged for 4 hours at 100,000 rpm, at 4°C, in the TL100 machine

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comprising a TL100.4 rotor. The resulting upper fraction containing the LDLs is recovered and stored at 4°C.

5 The various fractions harvested are then dialyzed for 18 hours at 4°C against a 0.15 M NaCl/0.24 mM EDTA buffer. The fractions are then recovered and filtered over a 0.45 µ membrane. A protein assay carried out by the Lowry method (Sigma).

10

Example 2: Cell culture

The culture medium used is DMEM medium (marketed by Gibco BRL) supplemented with: 1 mM of sodium pyruvate
15 (Gibco), 1% of nonessential amino acids (Boehringer Mannheim), 2 mM of glutamine (Gibco BRL), 200 U/ml of penicillin (bioMérieux), 200 mg/ml of streptomycin (bioMérieux) and 10% of calf serum (Boehringer).

20 The cells originate from the PLC/PRF/5 cell line (Alexander cells) (ATCC reference: CRL 8024), which is a line established from a human hepatocarcinoma. This line, which has integrated a defective hepatitis B genome, secretes the HBs antigen of the hepatitis B
25 virus but no infectious virus. The cells are cultured in the complete DMEM medium (Gibco-BRL) supplemented with 10% of foetal calf serum, mentioned above. The cells are washed with PBS (Gibco-BRL) preheated to 37°C. 1 ml of DMEM supplemented with 0.2% of BSA
30 (Sigma) and 50 µg/ml of lipoproteins originating from each fraction or 100 µl of serum filtered over a 0.45 µ membrane are added. Simultaneously, a solution of oleic acid in isopropanol, prepared beforehand (100 mM), is added in a concentration range of 0.1 to 0.8 mM. The
35 cells are then subjected to incubation for 3 hours at 37°C under a 5% CO₂ atmosphere. The medium is then replaced with complete DMEM medium supplemented with 10% of foetal calf serum. The cells are returned to the incubator under the conditions described above. The

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culture supernatants are regularly removed in order to serve, by RT-PCR, for the genome of the hepatitis C virus. In parallel, an analysis by immunofluorescence is carried out on the infected cells and controlled
5 using an anti-HCV structural protein monoclonal antibody.

Example 3: Results

- 10 The study is carried out using various LVP fractions obtained from plasma of two patients infected with HCV. The presence of the HCV genome in these various fractions was demonstrated by semi-nested RT-PCR.

- 20 -

Patient	LVP fractions with a density d*:	1st round	2nd round
Patient No. 1	d < 1.0063	+	+
	1.063 < d < 1.0063	-	-
	d > 1.063	-	+
Patient No. 2	d < 1.0063	-	+
	1.063 < d < 1.0063	-	+
	d > 1.063	+	+

*: density d in g/ml

5 These results show the presence of a signal positive for the presence of the HCV genome in the fractions with a density of less than 1.0063 g/ml, in both patients, either in the first round of PCR, in the second round of PCR.

10

The analysis of the fraction with a density of between 1.063 and 1.0063 g/ml is negative for the presence of the HCV genome in patient number 1, but positive in the second round of PCR in patient number 2. The fraction
 15 with a density of greater than 1.063 g/ml is positive for the presence of the HCV genome in both patients, but the response is weaker for patient 1 than for patient 2. These differences between patient no. 1 and patient no. 2 in the fraction with a density of between
 20 1.063 and 1.0063 g/ml may be explained by a lower viral load in patient no. 1.

The results were obtained using culture supernatants after infection of 100 µl of serum taken from patients
 25 no. 1 and no. 2 in the presence or absence of 0.5 mM oleic acid. Negative controls were performed under the same conditions. The analysis performed by semi-nested RT-PCT shows that, for both patients, the presence of the HCV genome in the culture supernatants is detected
 30 at a higher frequency in the assays for which the infection was carried out in the presence of oleate.

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Similar assays, carried out using the fractions with different densities (less than 1.0063 g/ml, between 1.0063 g/ml and 1.063 g/ml and greater than 1.063 g/ml), confirm these results and show that oleic acid facilitates the pathway for endocytosis of the LVPs at the time of the infection.

Example 4: Study of the kinetics of attachment of the LVPs to the LSR receptor of hepatocytes in the presence or absence of oleate.

A fraction containing LVPs with a density of between 1.025 g/ml and 1.055 g/ml was obtained from an HCV-positive serum as described in example 1.

The amount of proteins present in the LVP fraction was determined by the Lowry method using the "Protein Assay" kit (Sigma Diagnostics) and the viral titer was evaluated by quantification of the HCV RNA by RT-PCR and real-time fluorescence detection (LightCycler™, Roche) (Wittwer et al., Biotechniques, 22: 176-181 (1997)).

The PLC/PRF/5 cell line, described in example 2 and cultured in DMEM medium-10% SVF, was used for studying the attachment of LVPs to the LSR receptor in the presence or absence of oleate.

The results were obtained according to the following protocol:

The PLC/PRF/5 cells were seeded at 1 million cells/well, so as to obtain, after incubation for 24 hours at 37°C, 5% CO₂, approximately 80% confluence.

Two washes in cold 1X PBS buffer were carried out, and then 1 ml of cold complete DMEM medium containing 0.2%

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of BSA is added to the PLC cells maintained on a bed of ice.

The LVP fraction (i.e. 10 mg of proteins and 1.7×10^6 of copies of HCV RNA) is added to the cells in the presence or absence of 0.5 mM oleate. All the assays were carried out in triplicate.

An incubation was carried out at +4°C for a period of time ranging from 1 to 9 hours. The cells were then washed with three times with cold 1X PBS, followed by lysees with 0.5 ml of lysis buffer from the Rneasy kit (Qiagen). The RNAs were then purified using this same kit and analyzed by quantitative RT-PCR (LightCycler™).

The results obtained are indicated in the attached figure, in which the incubation time is represented on the X-axis and the number of HCV RNA copies is represented on the Y-axis. In the graphic representation of this figure, the dark bars refer to a culture without oleate and the light bars refer to a culture with oleate. The results show that, in the presence of oleate, attachment of the LVPs to the LSR receptor is significantly increased compared with the assays without oleate.

Example 5: Demonstration of the association of the LVPs with human immunoglobulins.

Various fractions containing LVPs harvested from plasma of three patients infected with HCV and prepared according to the protocol of example 1 were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of SDS (sodium dodecyl sulfate) (SDS-PAGE) (Laemmli, Nature (1970), 227: 680-685). The presence of immunoglobulins (Igs) in these fractions was demonstrated using the Western Blot technique (Towbin et al., PNAS, (1979) 76: 4350-4354), using a peroxidase-coupled anti-human immunoglobulin goat serum

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	$d^* < 1.0063$	$d^* 1.063 < d > 1.0063$
Presence of Igs	+++	+/-
Quantification of RNAs (per 0.2 ml of LVPs)	32400 copies	235800 copies
Quantification of RNAs copurified with Ig	21300 copies (65.7%)	21300 copies (9%)

d^* signifies the density of the LVPs in g/ml.

Patent No. 3

	$d^* < 1.0063$	$d^* 1.025 < d > 1.0055$
Presence of Igs	+++	+
Quantification of RNAs (per 0.2 ml of LVPs)	197100 copies	45900 copies
Quantification of RNAs copurified with Ig	142500 copies (72.3%)	26100 copies (56.8%)

d^* signifies the density of the LVPs in g/ml.

5

These results show that, when immunoglobulins are present in the fractions containing LVPs, the viral RNAs are mainly found in the fractions of LVPs associated with human immunoglobulins.

10

CLAIMS

1. A method for culturing, propagating and replicating, *in vitro*, viruses belonging to the *Togaviridae* or *Flaviviridae* families, according to which there is at least one LVP fraction obtained from serum or from plasma of a patient infected with at least one virus belonging to the *Togaviridae* or *Flaviviridae* families, and said fraction is brought into contact, for a predetermined period of time in a suitable culture medium, with permissible cells which have an endocytosis pathway relayed by at least one receptor for lipoproteins and modulated by an activating agent chosen from an unsaturated fatty acid, or a derivative of an unsaturated fatty acid comprising from 16 to 20 carbon atoms or a mixture thereof.
2. A method for culturing, propagating and replicating, *in vitro*, viruses belonging to the *Togaviridae* or *Flaviviridae* families, according to which there is at least one LVP fraction, associated with human immunoglobulins, obtained from serum or from plasma of a patient infected with at least one virus belonging to the *Togaviridae* or *Flaviviridae* families, and said fraction is brought into contact, for a predetermined period of time in a suitable culture medium, with permissible cells which have an endocytosis pathway relayed by at least one receptor for lipoproteins and modulated by an activating agent chosen from an unsaturated fatty acid, or a derivative of an unsaturated fatty acid comprising from 16 to 20 carbon atoms or a mixture thereof.

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3. The method as claimed in claim 1 or 2, in which the receptor for lipoproteins is the LSR and/or the surface receptor for LDLs.
- 5 4. The method as claimed in any of the preceding claims, in which the unsaturated fatty acid is chosen from oleic acid, palmitoleic acid, linoleic acid, linolenic acid, arachidonic acid, transhexadecenoic acid and elaidic acid, or
10 derivatives thereof.
5. The method as claimed in claim 4, in which the fatty acid is oleic acid, which is added to said culture medium at a concentration of between 0.1
15 and 1 mM, preferably 0.5 mM.
6. The method as claimed in any one of the preceding claims, in which the permissive cells are primary human or animal hepatocyte cells, cells chosen
20 from the human or animal hepatocarcinoma cell line group, dendritic cells, macrophage cells, Kupffer cells and combinations thereof which may or may not be associated with lymphocytes.
- 25 7. The method as claimed in claim 6, in which the permissives cells are human hepatocarcinoma cells of the PLC/PRF/5 cell line.
8. The method as claimed in any one of the preceding
30 claims, in which the culture medium comprises, besides the ingredients required for culturing and the fatty acid or the derivative of fatty acid, an apoptosis-modulating agent.
- 35 9. The method as claimed in claim 8, in which the apoptosis-modulating agent is chosen from interferons, anti-interferons, in particular anti-alpha and beta interferons; anti-caspases 3, in

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16. A medium for culturing, propagating and replicating at least one virus belonging to the Togaviridae and Flaviviridae families, said medium
5 being the BMEM medium supplemented with 0 to 10 mM of sodium pyruvate, 0 to 10% of nonessential amino acids, 1 to 10 mM of glutamine, 100 to 200 U/ml of penicillin, 100 to 200 mg/ml of streptomycin and 1 to 20% of calf
10 serum, and advantageously also comprising 0.1 to 0.5% of BSA or HSA coupled to a fatty acid.
17. A method for preparing a composition for detecting, in a sample, antibodies directed
15 against at least one virus belonging to the *Togaviridae* and *Flaviviridae* families, which comprises at least one partial or total purification of the viral particles of said virus or of the polypeptides obtained in using a
20 culturing method as claimed in claim 1 or 2.
18. The method as claimed in claim 17, in which said viral particles or said polypeptides are attached
25 to a solid support.
19. A method for obtaining antibodies or antibody fragments directed against at least one virus belonging to the *Togaviridae* and *Flaviviridae* families, according to which an animal is
30 immunized with viral particles or polypeptides obtained using a culturing method as claimed in claim 1 or 2.
20. A diagnostic composition comprising at least the
35 viral particles or the polypeptides obtained according to the method defined in either of claims 17 and 18 or the antibodies obtained according to the method defined in claim 19.

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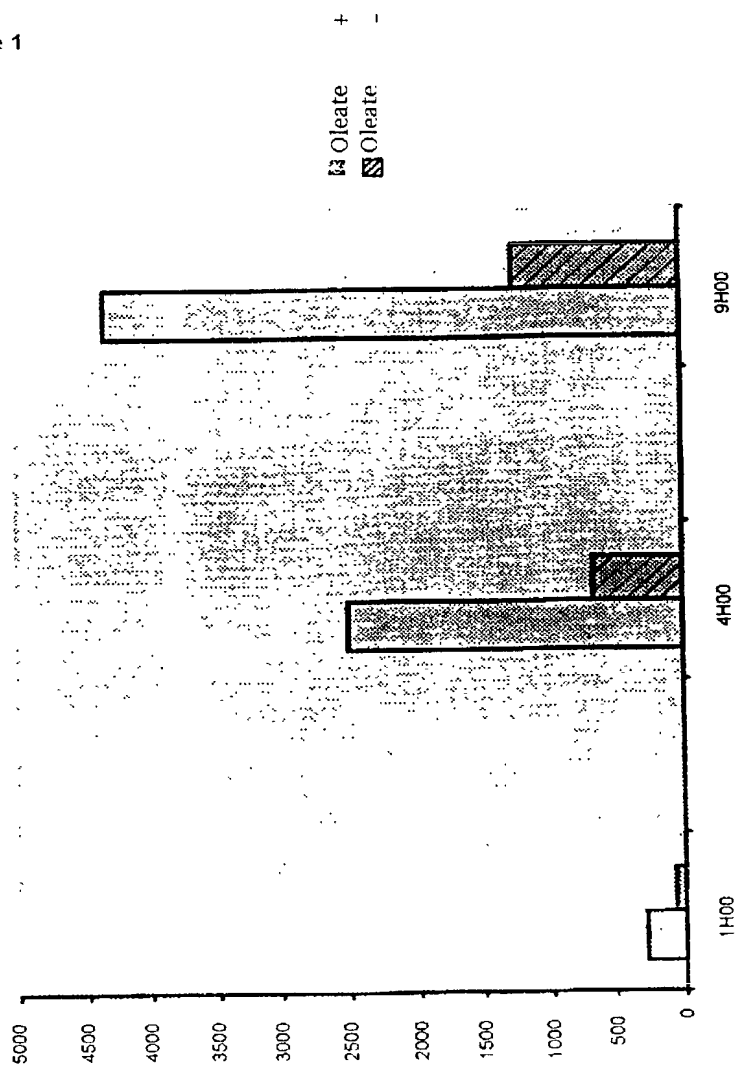
21. A diagnostic kit also comprising a composition as defined in claim 20.
- 5 22. An immunization composition comprising at least the viral particles of the polypeptides obtained according to the method defined in claim 17, associated with a pharmaceutically acceptable vehicle and/or excipient and/or adjuvant.
- 10 23. A therapeutic composition capable of qualitatively and/or quantitatively influencing the propagation and replication, *in vivo*, of viruses belonging to the *Togaviridae* and *Flaviviridae* families, which comprises, inter alia, a ligand capable of
15 modulating, of repressing or of inhibiting the endocytosis pathway relayed by at least receptors for lipoproteins, the ligand being chosen from an antagonistic antibody directed against said receptor and a protein chosen from soluble
20 recombinant proteins and soluble synthetic polypeptides, which bind said receptor, or in that it comprises, inter alia, at least one molecule which modulates, represses or inhibits the
25 expression of the gene encoding said receptor or the activity of the promoter of the gene which encodes said receptor.
- 30 24. A method for screening and/or selecting at least one antiviral molecule, according to which said antiviral molecule is brought into contact with an infected cell line.

ABSTRACT

The invention concerns a method of culturing, propagating and replicating in vitro viruses of the *Togaviridae* and *Flaviviridae* families which consists in: providing at least a fraction of LVP's obtained from serum or plasma of a patient infected with at least a virus of the *Togaviridae* and *Flaviviridae* families; contacting said fraction, for a predetermined time interval in an appropriate culture medium, with permissive cells having an endocytosis path relayed by at least one receptor of the lipoproteins and modulated inter alia by an activating agent selected among an unsaturated fatty acid or an unsaturated fatty acid derivative comprising 16 to 20 carbon atoms or their mixture. The invention also concerns the uses of viral particles and polypeptides obtained by said method.

1/1

Figure 1



**DECLARATION AND POWER OF ATTORNEY
UNDER 35 USC §371(c)(4) FOR
PCT APPLICATION FOR UNITED STATES PATENT**

As a below named inventor, I hereby declare that:
my residence, post office address and citizenship are as stated below under my name;

I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought, namely the invention entitled: METHOD FOR IN VITRO CULTURE OF VIRUSES OF THE TOGAVIRIDAE AND FLAVIVIRIDAE FAMILIES AND USES

described and claimed in international application number PCT/FR00/02202 filed July 31, 2000. ✓

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations §1.56.

Under Title 35, U.S. Code §119, the priority benefits of the following foreign application(s) filed by me or my legal representatives or assigns within one year prior to my international application are hereby claimed:

French Patent Application No 99 10095 filed July 30, 1999. ✓

The following application(s) for patent or inventor's certificate on this invention were filed in countries foreign to the United States of America either (a) more than one year prior to my international application, or (b) before the filing date of the above-named foreign priority application(s):

I hereby appoint the following as my attorneys of record with full power of substitution and revocation to prosecute this application and to transact all business in the Patent Office:

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ALL CORRESPONDENCE IN CONNECTION WITH THIS APPLICATION SHOULD BE SENT TO OLIFF & BERRIDGE, PLC, P.O. BOX 19928, ALEXANDRIA, VIRGINIA 22320, TELEPHONE (703) 836-6400.

I hereby declare that I have reviewed and understand the contents of this Declaration, and that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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3 **Date of Signature:**

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